Prallethrin-Induced Excitation Increases Contact Between Sprayed Ultralow Volume Droplets and Flying Mosquitoes (Diptera: Culicidae) in a Wind Tunnel

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ABSTRACT Female *Culex quinquefasciatus* Say (Diptera: Culicidae) mosquitoes were exposed to sublethal amounts of prallethrin, sumithrin, and piperonyl butoxide applied as ultralow volume (ULV) droplets in a wind tunnel. Mosquitoes were video recorded before, during, and after treatment, and the number and size of droplets on their bodies were later determined using a compound microscope. A positive correlation was found between mosquitoes that spent more time flying during the time of spraying and number of droplets on their bodies. Excitation, in the form of increased speed and duration of flight, was immediate in mosquitoes exposed to prallethrin, whereas exposure to sumithrin did not increase their exposure to the ULV droplets. The location of droplets on mosquitoes, the effects of droplet volume, and subsequent mortality are discussed.

KEY WORDS ultralow volume droplets, excitation, mosquito behavior, wind tunnel, pyrethroids

Ultralow volume (ULV) application of insecticides is a commonly accepted method for controlling or reducing populations of adult mosquitoes and has been extensively reviewed by Mount (1998). Such treatments are commonly applied using backpack or truck-mounted sprayers. To be effective against flying mosquitoes, and minimize nontarget effects, federal requirements dictate the droplet spectrum of ULV applications needs to have a volume median diameter (VMD) of <30 μm, and at least 90% of the spray must be contained in droplets <50 μm for ground-based applications (USEPA 2006). For each product, droplet parameters are specified by the manufacturer with the majority of droplets occurring within the above-mentioned range so that they will remain airborne for extended durations, thereby maximizing the number of droplets that may impinge on mosquitoes to optimize mortality.

Previous work by Curtis and Mason (1988) and Rathburn and Dukes (1989) revealed that the size, number, and volume of droplets that penetrate densely vegetated areas is lower than in open areas, resulting in reduced efficacy of ULV insecticide applications. Indeed, Rathburn and Dukes (1989)

showed a 2.5-fold reduction in droplets occurred in vegetated areas compared with open areas that resulted in a corresponding 10-fold reduction in mosquito mortality. Rathburn and Dukes (1989) further stated that to achieve the same mortality in dense vegetation as in open areas the application rate would have to be increased severalfold (Rathburn and Dukes 1989).

The movement of tiny objects, such as ULV droplets, through a fluid (e.g., air) is dictated by the velocity and length of the object, and the viscosity of the fluid it is moving through. When tiny objects approach larger surfaces they encounter increased drag as they enter the boundary layer (the layer of fluid adjacent to the object which resists moving with respect to that object) (Loudon et al. 1994). Boundary layers in densely vegetated areas reduce air movement and the movement of smaller objects (such as ULV droplets) among vegetation (Patel et al. 1985). As a result, mosquitoes resting on vegetation, or other protected surfaces, are more protected from ULV droplets and more likely to receive a sublethal dose, whereas airborne droplets are more likely to impinge upon flying mosquitoes. If this assumption is correct, it would be advantageous during ULV applications to use chemicals that cause excitation and flush mosquitoes from hiding places into the aerosol application.

The pyrethroid insecticide sumithrin is used as a mosquito adulticide in control programs worldwide (Garcia et al. 2009). It is an effective insecticide but is not particularly known for its excitatory properties. Prallethrin is a relatively volatile pyrethroid with repellent properties against mosquitoes. Moreover, this

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14. ABSTRACT
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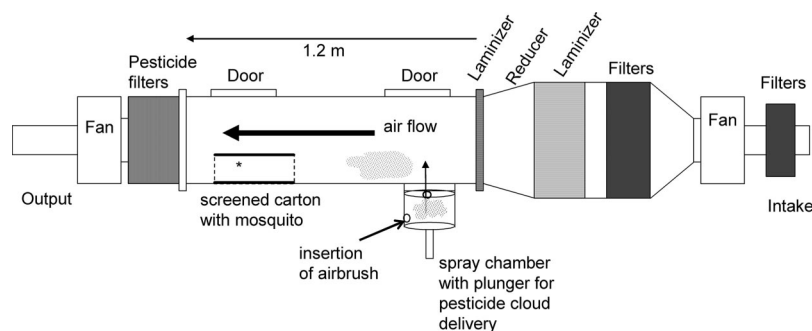Prescribed by ANSI Std Z39-18

Fig. 1. Schematic diagram of the side view of the wind tunnel.

pyrethroid is thought to have a strong excitatory component (Matsunaga et al. 1987, Groves et al. 1997, Emmrich et al. 2003). These properties make prallethrin a good candidate as a flushing agent. The objective of this study was to test proof of concept that female mosquitoes when exposed to droplets of prallethrin resulted in increased excitation and increased droplet contact with increased mortality.

Materials and Methods

Insects. Mosquitoes used in this experiment were from a laboratory colony of *Cx. quinquefasciatus* from Gainesville, FL, maintained since 1995 by using methods described by Gerberg et al. (1994) and supplemented with field-collected specimens every 1–2 yr. Adults were maintained in BugDorm-1 (30-cm^3 Mega-View Science Co., Ltd., Taichung, Taiwan) cages in an environmental chamber set at 27°C, 70% RH, and a photoperiod of 16:8 (L:D) h. Before bioassays, newly emerged adults were held for 4–8 d to allow for mating and maturation and were provided with 10% sugar water solution. A battery-operated aspirator (Hausherr's Machine Works, Toms River, NJ) was used to transfer female mosquitoes into a screened paper carton where they were held for 30–150 min before being used in tests.

Wind Tunnel. A push-pull Plexiglas wind tunnel with a working section of 30 by 30 by 120 cm was designed for this experiment (Fig. 1). An upwind and downwind door on the top of the wind tunnel allowed for access; the entire top of the working section also could be removed. The bottom of the wind tunnel contained upwind and downwind slots for removable attachment panels which could be solid, have a hole for insertion of an anemometer, or an attached spray chamber for the controlled release of insecticide into the wind tunnel.

The spray chamber consisted of a cylinder (15.6 cm in diameter by 16.2 cm in height), with a plunger. The tip of an airbrush nozzle (see below) was inserted into a 1.6-cm hole, located 1.9 cm from the bottom of the cylinder (adhesive tape covered the hole when not in use). The inner top surface of the spray chamber contained a 1.3-cm hole with a flap of acetate hanging from it which acted as a valve. The acetate valve sealed the hole while the airbrush was sprayed but remained

open while the plunger was slowly pumped, allowing the spray cloud to enter the tunnel in a controlled manner. Each treatment had its own spray chamber to minimize contamination between treatments.

The wind tunnel was lined with aluminum foil and plastic wrap to prevent contamination between each treatment. Aluminum foil was used to line the floor and back walls of the wind tunnel, whereas plastic wrap was used along the front and top walls.

A hotwire anemometer (TSI, Inc., Shoreview, MN) was used to measure the airflow in the wind tunnel and inside the screened cage. The wind speed in the tunnel was adjusted to 100 cm/s to attain wind speed within the screened cage of ≈50 cm/s, high enough to uniformly carry droplets into the cage (Hoffmann et al. 2008). Under these conditions, the air in the tunnel was completely replaced every 1.2 s. The average temperature and humidity during experiments was 24.8°C and 69.2%, respectively, whereas the light level inside the screened cage was 106.8 lux.

Airbrush Calibration. A single action airbrush (model 350, Badger, Franklin Park, IL) was used to produce the ULV droplets. The airbrush was tested with DUET Dual-action Adulticide (hereafter referred to as Duet) (Clarke Mosquito Control, Roselle, IL) with various nozzle sizes and pressure settings to determine which configuration produced droplets that conformed best to the label instructions for Duet. Tests of settings were repeated two times. A plastic settling chamber was constructed (36.8 cm in width by 30.5 cm in depth by 68.6 cm in height) with a hole for insertion of the airbrush nozzle 57.2 cm above a Teflon-coated glass microscope slide. The airbrush was sprayed for 0.5 s into the settling chamber, and droplets were allowed to settle for ≈10 s (Brown et al. 1990). Droplets were measured under a compound microscope equipped with a 40× objective, and 10× ocular lens with a 10-mm reticule with 0.1-mm divisions. A stage micrometer was used to determine that each eyepiece division was 2.5 μm. During the behavioral bioassay, a nozzle equipped with a heavy tip and fine needle was used, and the air pressure was set to 40 psi.

Bioassay Procedures. One quart (0.95 liters) cardboard milk cartons were modified for use in the bioassays. The cartons had nylon tulle mesh (with 0.075-mm fiber width and 1-mm^2 openings) glued to

both ends, so that air could enter the mesh and flow through the length of the carton. The side of the carton facing the video camera was cut out and replaced with a sheet of acetate to form a window. Finally, a hole was cut out of the bottom of the carton (the bottom defined as the side that would lie on the floor of the wind tunnel), so that an individual mosquito could be placed in and removed from the carton easily, and through which the anemometer could be inserted via a hole in the wind tunnel's replaceable floor panel for measuring wind speed within the carton. A new carton was used for each mosquito to avoid contamination.

One carton, containing a female *Cx. quinquefasciatus* mosquito (4–8 d old), was placed at the downwind end of the wind tunnel. A preliminary test determined that the test compounds would be sprayed into the spray chamber for 0.5 s to expose mosquitoes to a 24-h sublethal dose. A video camera (model WV-BP334, Panasonic, Sezhou, China) with automatic iris lens (CCTV, 1/3 in., 3–8 mm, F 1.4, Rainbow, Irvine, CA) connected to a digital MPEG recorder (model EMR100, Canopus Co., Kobe, Japan) that was connected to a laptop computer captured the bioassay videos in MPEG2 format at 30 frames per s. Power-Director software version 6 (Cyberlink, Taipei, Taiwan) was used to prepare digital videos for analysis with Observer XT version 7 (Noldus Information Technologies, Wageningen, The Netherlands) and Motus version 8.2 (Peak Performance Technologies, Inc., Centennial, CO) by reducing them from 30 to five frames per s, improving contrast and brightness, and cutting files to make clips of desired video segments.

Each recording had three segments: prespray, spray, and postspray. The prespray segment served as a control baseline for each mosquito and consisted of the mosquito being recorded for 5 min before spraying. A timer was used to time when the prespray segment ended and the spray segment commenced, at which time a piece of foil was passed in front of the camera lens to signal on the video record that spraying was about to begin. At the commencement of the spray segment, the airbrush trigger was pressed for 0.5 s, as measured using a digital stopwatch. Once spraying was complete, working quickly, one person removed the airbrush tip from the hole while a second person immediately covered the hole with a piece of tape. The spray chamber was then slowly plunged so that the droplets of the treatment inside were displaced into the wind tunnel. Each plunge took approximately thirty seconds to complete, and the spray chamber was plunged five times, for a total plunging time of 2.5 min. Once the fifth plunge was complete, a piece of foil was again passed in front of the camera lens to signal on the video record that spraying and plunging were complete. Recording continued for an additional 5-min postspray period, bringing the total recording time to ≈12.5 min.

Five treatments consisting of synergized prallethrin, sumithrin, and their mixture (a commercial formulation of the adulticide Duet) were evaluated in this study (Table 1). All compounds were diluted in

Table 1. Components of the four treatments and control evaluated against *Cx. quinquefasciatus* in laboratory wind tunnel bioassays[a]

Content	Treatment (%)				
	A	B	C	D (Duet)	E (control)
Prallethrin	−	1	1	1	−
Sumithrin	5	−	−	5	−
PBO	5	−	5	5	−
Inert ingredients	+	+	+	+	+

[a] Dash (−) indicates that ingredient was absent; and plus (+) indicates that the inert ingredients of Duet, a proprietary mixture containing hydrotreated paraffinic oil and aromatic hydrocarbons, make up the balance of the solution.

the same proprietary inert mixture of hydrotreated paraffinic oil and aromatic hydrocarbons used in Duet and were provided by Clarke Mosquito Control. Ten replicates were conducted for each of the five treatments. In between each replicate, the spray chamber was plunged several times to clear out all droplets that may have remained in the chamber. Five replicates of the same treatment were conducted using a new mosquito carton each time, then the entire lining of the wind tunnel was changed and five replicates of the next treatment were conducted. The number of replicates and order of treatments were as follows: 5E, 5D, 5C, 5B, 5A, and then this sequence of events was repeated. Between treatments the airbrush was dismantled and its parts rinsed in acetone five times. Before reusing the airbrush, clean acetone was sprayed through it five times into a separate spray chamber.

Mortality. As soon as a video recording was completed, the cardboard carton was removed from the wind tunnel, and the mosquito was transferred by aspiration into a clean, screened paper carton with no food or water. The mosquito was observed every 30 min for 8 h and once after 24 h to quantify mortality. Knockdown (KD) was defined as the mosquito lying on the floor of the carton, either on its side or back, or upright but not in a standing posture. Death was defined as a knocked down mosquito that was unresponsive if blown upon gently. Abbott's formula (Abbott 1925) was applied to compensate for any mortality found in controls.

Quantification of Droplets. After mortality was recorded, all mosquitoes were stored at −12°C. Then, each mosquito was dismembered and all appendages and parts were mounted onto clear double sticky tape on a microscope slide. Each wing, leg, antenna, and the head, thorax, and abdomen was examined for droplets by using the compound microscope, as described above. The location and diameter of each droplet was recorded, and the total droplet number and volume were later calculated using the formula for the volume of a sphere, because the spread factor is unknown for the surface of a mosquito, and droplets seemed to remain somewhat spherical (Fig. 2).

Data Acquisition From Videos. For each video recording, a frame-by-frame analysis was conducted in which the two-dimensional (2-D) position was digi-
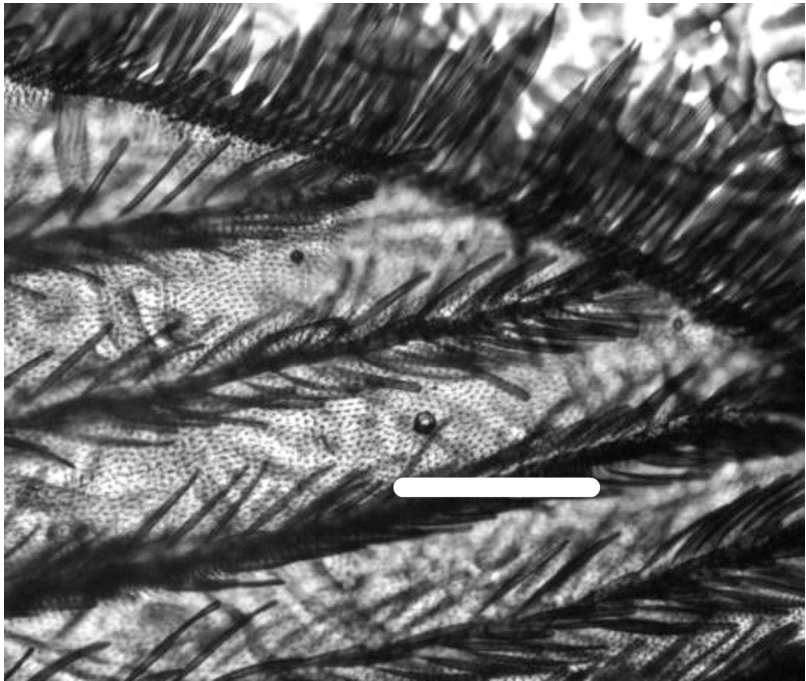
Fig. 2. Droplet on the wing of a mosquito. White scale bar = 50 μm.

tally entered into Motus, which then calculated x- and y-coordinates for each frame. These coordinates were exported to an Excel (Microsoft, Redmond, WA) spreadsheet that was used to calculate 2-D vectors, distance traveled, velocity, and percentage of time spent in motion (percent movement) in each segment.

Videos were examined a second time to quantify the amount of time mosquitoes spent in each behavior (e.g., walking, flying, resting, knockdown) by using Observer XT for each of the three video segments for each mosquito. The percentage of time mosquitoes spent flying divided by the total time was calculated for each video segment.

Statistical Analysis. The study used a double-blind design. Means of percentage of movement (i.e., walking and flying) were not normally distributed, however, when means in each time period were subtracted from the other time periods, differences were normally distributed and could then be analyzed using proc GLM and Tukey means separation test (SAS version 9.2, SAS Institute, Cary, NC), with the α rejection region of 0.025 rather than 0.05, due to using each data set in two paired comparisons and subsequent Bonferroni correction of the critical alpha. In each time segment, the Dunnett's test was used to compare each formulation to the control treatment for average velocity and percentage of time spent flying. For each of the three time segments, Pearson's correlation coefficients were calculated by plotting the number of droplets against the percent of time spent flying using PROC CORR (SAS Institute). Correlation analyses were also conducted between volume and

droplet number, as well as volume and percentage of time flying.

Results

Droplet Spectra. The various airbrush configurations resulted in different droplet spectra (Table 2). The Duet label states that "spray equipment must be adjusted so the volume median diameter (VMD) is between 8 and 30 μm (DV 0.5 < 30) and that 90% of the spray is in droplets <50 μm (DV 0.9 < 50 μm)." Using a heavy tip and a fine needle, and adjusting air pressure to 40 psi produced a mean VMD of 9.2 μm, a range of 1.6–37.2 μm, with 3% of droplets >30 μm. These parameters were the closest to the label recommendations for Duet and were used throughout

Table 2. Airbrush configurations and their resulting droplet spectra

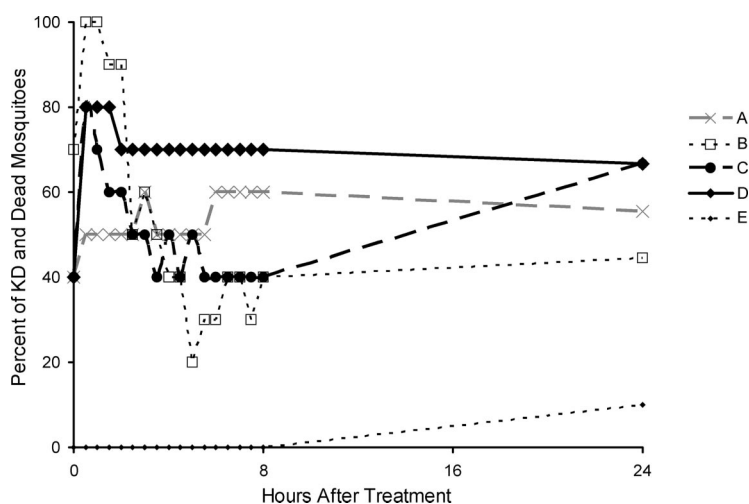| Tip	Needle	Air pressure (psi)	VMD (μm)	Range (μm)	% >30 μm
Fine	Fine	30	21.9	1.6–76.0	14.0
Fine	Fine	35	21.3	1.6–86.8	13.0
Fine	Fine	40	16.5	1.6–51.2	7.3
Fine	Fine	45	16.7	1.6–54.3	5.8
Fine	Fine	50	12.7	1.6–45.0	4.5
Fine	Medium	50	14.9	1.6–49.6	5.0
Medium	Fine	50	13.3	1.6–62.0	4.3
Medium	Medium	30	25.6	3.1–76.0	18.8
Heavy	Fine	30	13.6	1.6–46.5	2.0
Heavy	Fine	40	9.2	1.6–37.2	3.0
Heavy	Fine	50	9.0	1.6–32.6	0.5
Heavy	Large	30	22.3	1.6–103.9	15.0

Fig. 3. Total percentage of KD and dead mosquitoes in each treatment over time. A, Sumithrin, PBO, and inert ingredients; B, prallethrin and inert ingredients; C, prallethrin, PBO, and inert ingredients; D, sumithrin, prallethrin, PBO, and inert ingredients (Duet); and E, only inert ingredients (control).

the experiment. To simulate spraying a mosquito through a cage, a Teflon-coated slide in the settling chamber was placed inside a mesh cage and the same airbrush settings were used ($n = 5$), resulting in a VMD of 4.9, with a range of 1.6–15.9 μm, and 0% of droplets >30 μm. Spraying caged mosquitoes in the settling chamber with these settings for 0.5 s, and then removing them after 10 s resulted in a sublethal dose of Duet after 24 h.

Mortality. More KD and recovery was seen with treatments B and C (prallethrin only, and prallethrin plus piperonyl butoxide [PBO], respectively), whereas the greatest mortality with least recovery from KD was treatment D (Duet) (Fig. 3).

Droplets. The majority of the observed droplets were found on the legs and wings of mosquitoes (Fig. 4), and most of the droplets per mosquito measured 2.5–5 μm in diameter (Fig. 5). No differences were found between treatments in number and size of droplets.

Movement. Mosquitoes exposed to treatment D (Duet) that died, showed more movement during spray than those that survived (Table 3; Fig. 6). Mos-

quitoes exposed to treatment A (sumithrin and PBO) that died, showed more movement after spraying was over than those that survived.

Mosquitoes in treatments D (Duet) and B (prallethrin) spent significantly more time in flight during the spray period than the control mosquitoes, whereas mosquitoes in treatments A (sumithrin plus PBO) and C (prallethrin plus PBO) did not spend more time in flight during the spray period compared with control mosquitoes (Fig. 7).

During the spray period, velocity of mosquitoes was greater when exposed to formulations containing prallethrin (treatments B–D) than the control. Mosquitoes exposed to the sumithrin and PBO formulation (treatment A) did not move significantly faster than control mosquitoes during the spray period (Fig. 8). For all four treatments mosquitoes moved faster than control mosquitoes during the postspray period. There were no differences before spraying between any of the treatments and controls, indicating the bioassay was free of contamination (Figs. 7 and 8).

Correlations. There was no significant correlation between droplet number and droplet volume, nor between droplet volume and percent of time spent flying in the three video segments. There was a sig-
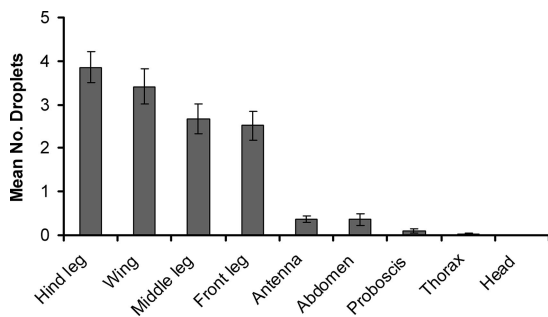
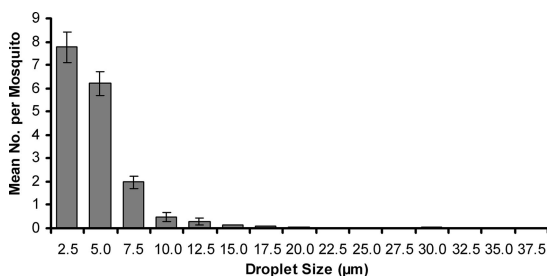**Fig. 4.** Mean number of droplets counted on different body parts of mosquitoes.

Fig. 5. Average number of droplets of different sizes (micrometers) found per mosquito.

Table 3. Analysis of variance table comparing differences in the percentage of time spent moving (sum of both walking and flying), from one time segment to another, of mosquitoes that lived to those that died

Between time segments	Treatment[a]	df	F	P[b]
Pre to spray	A	1	4.54	0.0657
	B	1	4.29	0.0720
	C	1	2.83	0.1308
	D	1	17.35	0.0031
	E	0		
Pre to post	A	1	9.16	0.0164
	B	1	3.34	0.1050
	C	1	0.53	0.4862
	D	1	0.46	0.5167
	E	0		
Spray to post	A	1	0.19	0.6782
	B	1	5.88	0.0415
	C	1	2.87	0.1287
	D	1	15.72	0.0041
	E	0		

[a] A, Sumithrin, PBO, and inert ingredients; B, prallethrin and inert ingredients; C, prallethrin, PBO, and inert ingredients; D, sumithrin, prallethrin, PBO, and inert ingredients (Duet); and E, only inert ingredients (control).

[b] P value of <0.025 indicates that there was a significant difference between the behavior of mosquitoes that lived and those that died in 8 h after the experiment.

Fig. 7. Percentage of time mosquitoes spent flying before (pre), during (spray), and after (post) being sprayed. Asterisks (*) over bars indicate the mean of that treatment was significantly different from the control (E) ($P = 0.05$; Dunnett's test); n.s. indicates that no significant differences were detected. The five treatments consisted of inert ingredients and the following: A, Sumithrin, PBO, and inert ingredients; B, prallethrin and inert ingredients; C, prallethrin, PBO, and inert ingredients; D, sumithrin, prallethrin, PBO, and inert ingredients (Duet); and E, only inert ingredients (control).

nificant correlation, however, between the number of droplets and the percent of time spent flying during the spray period ($r = 0.536$, $P < 0.0001$) but not during the prespray or postspray periods (Table 4). There was no significant relationship between walking and droplet number or volume, nor was there a significant correlation between speed and droplet number for any of the time segments.

Discussion

This study provides experimental evidence that female *Cx. quinquefasciatus* exposed to ULV droplets containing 1% prallethrin causes an immediate increase in flight activity and speed. Moreover, increased time spent flying while droplets are airborne correlates positively with the number of ULV droplets that impinge on mosquitoes.
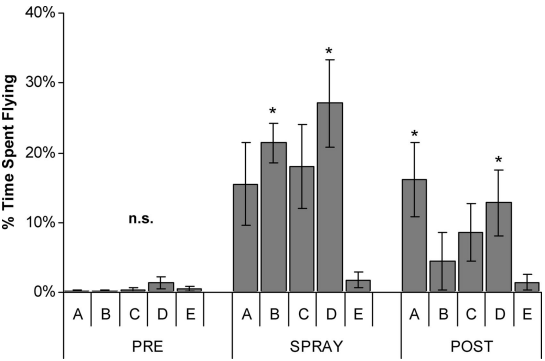
We found no correlation between volume (i.e., dose) and droplet number on mosquitoes because a single large droplet can have a much greater volume than several small droplets. Also, if an increase in locomotion was a result of an increase in dose, we would expect to see a correlation between volume and movement, but such a relationship did not exist. Instead, a significant positive correlation was found between movement and number of droplets, suggesting that mosquitoes picked up additional droplets as a result of being more active, rather than activity increasing as a result of increased dose. This suggests if more flight can be induced during the period when droplets are suspended in the air, the greater the likelihood that mosquitoes will come into contact with ULV droplets. Separating behavioral data between prespray, spray, and postspray intervals, was integral in detecting these patterns.

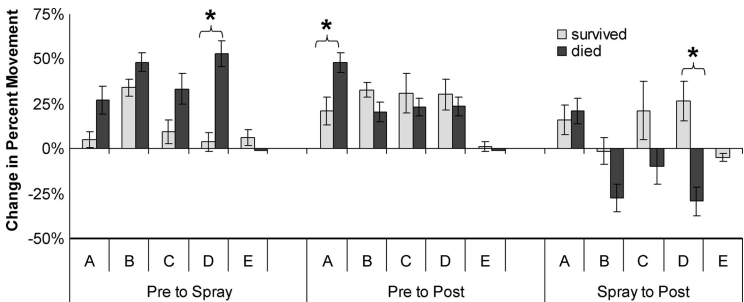Although relationships between treatment and flight and flight and droplet number were found, a

Fig. 6. Amount of change, from one time segment to another, of the percentage of time mosquitoes spent in motion (i.e., flying and walking). Asterisks (*) represent significant differences between mosquitoes that ultimately died versus those that lived in a particular treatment ($P < 0.025$; Tukey's test). The five treatments consisted of inert ingredients and the following: A, Sumithrin, PBO, and inert ingredients; B, prallethrin and inert ingredients; C, prallethrin, PBO, and inert ingredients; D, sumithrin, prallethrin, PBO, and inert ingredients (Duet); and E, only inert ingredients (control).
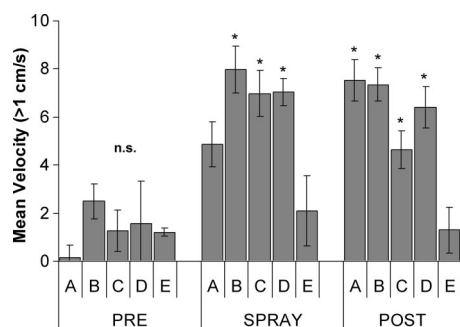
Fig. 8. Dunnett's test in each time segment comparing the mean velocity (>1 cm/s) of mosquitoes exposed to each formulation to the mean velocity of control mosquitoes. Asterisks (*) over bars indicate a significant difference from the control ($P > 0.05$); n.s. indicates that no significant differences were detected. The five treatments consisted of inert ingredients and the following: A, Sumithrin, PBO, and inert ingredients; B, prallethrin and inert ingredients; C, prallethrin, PBO, and inert ingredients; D, sumithrin, prallethrin, PBO, and inert ingredients (Duet); E, only inert ingredients (control).

direct relationship between treatment and number of droplets on mosquitoes was not found. This is probably due to the combination of the relatively small sample size, variations in individual insect behavior, their locations within the carton, and interactions with variations in size, movement and location of droplets within the carton that were not measured, tracked, or able to be controlled. Although considerable efforts were made to standardize the spray cloud, variations within the droplet clouds due to the formation of turbulent eddies as they traveled downwind, and the position of the mosquito within those eddies, would account for some degree of variation between individuals.

Size and number of droplets found on mosquitoes were similar to results obtained by Lofgren et al. (1973), however, in our study droplets were primarily found on the legs and wings, and rarely on the antennae. The fact that few droplets were found on the head, thorax, and abdomen may be due to the manner in which specimens were examined using a compound microscope, providing only a silhouette of thick body parts, so that only droplets located precisely on the edge were able to be observed.

Our results confirm that the two pyrethroids tested, prallethrin and sumithrin, are both locomotor stimulants as defined by Dethier et al. (1960) for *Cx. Quin-*

quefasciatus; however, excitation caused by prallethrin was more immediate than sumithrin. More work is needed to determine how the excitatory effects of prallethrin translate into mortality in the field. Because different species may not have the same behavioral responses to a given insecticide (Cooperband and Allan 2009), similar research on other species should be conducted to determine if the effects observed in our study are broadly applicable.

Acknowledgments

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Table 4. Pearson correlation coefficients and *P* values for percentage of time spent flying in the three video segments compared with number of droplets and droplet volume per mosquito after exposure ($n = 48$)

Behavior segment	No. droplets		Droplet vol.	
	Correlation	P	Correlation	P
Prespray	0.05183	0.7265	−0.09725	0.5108
Spray	0.53671	<0.0001	0.13302	0.3674
Postspray	−0.10675	0.4702	0.06139	0.6785

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